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Volume-sensitive chloride channels in the primary culture cells of human cervical carcinoma [1]

Meng-Ru Shen [a], Sheng-Nan Wu [b], Cheng-Yang Chou [a,*]

[a] *Department of Obstetrics and Gynecology, National Cheng Kung University Medical College, Tainan 704, Taiwan*
[b] *Department of Research and Education, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan*

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Abstract

Previous study shows volume-sensitive chloride currents are induced by hypotonicity in human cervical cancer cell lines, but not in normal cervical epithelium. To ascertain whether the preferential activation of these channels in cancer cell lines could be similarly and directly detected in cervical cancer tissues, we studied volume-sensitive chloride channels on the primary culture cells of invasive cervical carcinoma using the whole-cell patch-clamp technique. The process of regulatory volume decrease (RVD) was also studied using electronic cell sizing to measure cell volume. Results demonstrate that, in these cultured cells, RVD was mediated in part by chloride loss through the volume-sensitive Cl^- channels. A small background current with a slope conductance of 0.32 ± 0.07 nS/pF at +30 mV ($n = 60$ cells from 10 different samples) was observed. Hypotonicity induced a fast activating and outward rectifying current which was reversed at about 0 mV, and the slope conductance at +30 mV was increased by 10-fold to 3.62 ± 0.62 nS/pF. These effects were readily reversed by returning the cells to isotonic medium. Moreover, DIDS, NPPB, and 1,9-dideoxyforskolin, reversibly abolished the volume-sensitive Cl^- currents. The EC_{50} required for the inhibitory effect of DIDS, NPPB and 1,9-dideoxyforskolin was 150, 120, and 50 μM, respectively. Volume-sensitive Cl^- channels were ubiquitously expressed in cultured cells from 10 samples of different cancer stages, histopathologic types, and state of HPV DNA positivity. Interestingly, similar outward rectifying chloride currents were activated by intracellular 300 μM GTPγS. It is proposed that this Cl^- conductance may play an important role leading to RVD in human cervical cancer.

Keywords: Cervical carcinoma; Chloride channel; Volume regulation

1. Introduction

Volume regulation is a widespread process that enables cells to maintain their normal volume in the face of changes in extracellular osmolarity. An increase in cell volume activates membrane transport pathways that mediate an efflux of cell osmolytes to effect regulatory volume decrease (RVD) [1]. In one common type of RVD, KCl leaves the cell via distinct K^+ and Cl^- channels [2]. HeLa cells, a cervical adenocarcinoma cell line, develop large, volume-activated chloride currents on exposure to hypotonicity [3]. However, the expression of volume-sensitive chloride channels in squamous cell carcinoma of the uterine cervix, the major histologic type of cervical carcinoma, has not been studied. In the previous study [4], we found that hypotonicity activated an outward rectifying chloride current in all cervical cancer cell lines, but not in normal cervical epithelial cells. In addition, the activation of this current depended on intracellular ATP.

Because cervical cancer cell lines are relatively inexpensive, and readily available compared with cancer tissues, they provide a useful tool for the study of cervical carcinoma; however, a cell line is composed of a subset of cells with selective advantage in culture condition, and may not have the same eletrophysiological characteristics of cancer cells in vivo. To ascertain whether the preferential activation of these channels in cervical cancer cell lines could be detected in a similar fashion in cervical cancer tissues, we studied volume-sensitive Cl^- channels on the primary culture cells from 10 cases of invasive cervical carcinoma. The process of RVD was also studied. Our results demonstrate that RVD was mediated in part by

* Corresponding author. Fax: +886 6-2766185
[1] M.R.S. and S.N.W. contributed equally to this work.

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chloride loss through the volume-sensitive Cl^- channels in the primary culture cells of cervical carcinoma. Cell swelling activates the ATP-dependent Cl^- currents which were ubiquitously expressed in samples of various cancer stages, histopathological types, and HPV DNA positivity. Furthermore, G-protein seems to be involved in the activation of volume-sensitive Cl^- channel in cervical carcinoma.

2. Materials and methods

Establishment of primary culture and histopathological diagnosis. For explant culture of cervical carcinoma, tissue fragments of about 10 mm^3 in size were obtained from colposcopy-guided cervical biopsy to minimize the contamination of normal cervical epithelium at the department of Obstetrics and Gynecology, National Cheng Kung University, Tainan, Taiwan. Each fragment was cut into smaller pieces with a sterile scalpel, and were washed with sterile HBSS to remove contaminating RBC. Washed fragments were cultured in a 35-mm Petri dish in DMEM supplemented with 10% fetal bovine serum, and antibiotics at 37°C in a humidified 5% CO_2 incubator. When the explant cells began to appear on the edges of the tissue fragments, they were fed with fresh medium twice a week. Within two weeks, the primary explants were ready to use. The histopathological diagnosis of cervical carcinoma was confirmed on hematoxylin- and eosin-stained slides from the hysterectomized specimen. Furthermore, the cultured cells used for electrophysiological analysis were also stained with hematoxylin and eosin to rule out the contamination of stromal cells.

Detection and typing of HPV genome. For HPV detection and typing, the genomic DNA of each cell was amplified and type-classified using polymerase chain reaction (PCR) as described previously [5]. To reduce the false negative rate, two sets of primers were used. One set (MY09 and MY11) purchased from Perkin-Elmer, Cetus Corp. (Roche Molecular Systems, Branchburg, NJ, USA), was designed to amplify the L1 region from HPV types 6, 11, 16, 18, 31, 33 and at least other 25 HPV types [6]. The other set was the sense primer pU-1M, 5′-TGTCAAAAACCGTTGTGTCC-3′, sense primer pU-31B, 5′-TGCTAATTCGGTGCTACCTG-3′ and antisense primer pU-2R, 5′-GAGCTGTCGCTTAATTGCTC-3′. The pU-1M/pU-2R were used to amplify the E6/E7 region of HPV6 and 11, while pU-31B/pU-2R enabled amplification of the E6/E7 region of HPV 16, 18, 31, 33, 52b and 58 [7]. A positive and negative control were included in PCR, and r-interferon gene was used to evaluate DNA quality.

Measurements of cell volume. Cell volume was measured by electronic cell sizing as described in detail previously [8]. Briefly, cells were grown on tissue culture flask and were harvested by trypsinization. The 3×10^6 cells were suspended in 4 ml of an isotonic (300 mOsm/l) solution for 30 min [in mM: NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53. glucose 5.5, Hepes-NaCl buffer 5 (pH 7.4)]. Then, 500-μl aliquots of the cells in the isotonic solution were suspended in 20 ml of the isotonic solution, the hypotonic solution, or the hypotonic solution with 1 mM DIDS. The composition of hypotonic solution (200 mOsm/l) was as follows (in mM): NaCl 86, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, Hepes-NaOH buffer 5 (pH 7.4). Cell volume was measured for each condition at 100-s intervals till 15 min using a Coulter counter equipped with a Channelyzer (Coulter Corporation; Hialeah, FL, USA) and a counting probe with a 100-μm aperture. All experiments were done at 25°C.

Electrophysiological measurements. Membrane currents were measured with the whole-cell configuration on the non-confluent cells of cervical carcinoma. In order to monitor the change of cell size, the microscope was coupled to a video camera system with magnification up to 1500 ×. Cells were bathed at room temperature (20–25°C) and continuously superfused at a rate of about 2 ml/min with Tyrode's solution. Membrane potentials or ionic currents were recorded in current-clamp or voltage-clamp mode with patch pipettes in a whole-cell configuration as described [9,10]. For the preparation of the patch pipettes, the Kimax glass capillaries (Kimble Products, Vineland, New Jersey, USA) were heated and pulled by gravity using a two-step, vertical micropipette puller (PB-7; Narishige Scientific Instruments, Tokyo, Japan). When the pipettes were connected to the input stage of an Axopatch-1D amplifier (Axon Instruments, Burlingame, CA, USA), their DC resistance varied between 3 and 5 MΩ. A three-dimensional oil-driven micromanipulator (MO-103; Narishige Scientific Instruments, Tokyo, Japan), which was mounted on the fixed stage of an inverted microscope, was used to position the pipette near the cell. The liquid junctional potential was corrected immediately before the pipette was attached to the cell. In order to validate the experimental conditions, when the whole-cell recording mode was established, some cells were allowed to remain stabilized for about 5–10 min. In experiments designed to construct the current versus voltage (I-V) relationships, either square command pulses with a duration of 30 ms from the holding potential to various potentials or ramp command pulses with a duration of 100 ms from −80 mV to +40 mV were employed. The square or ramp voltage-step command signals were generated at a rate of 0.2–0.5 Hz by the use of a programmable stimulator (SMP 310; Biologic Corp., Claix, France).

Data recording and analysis. The signals consisting of voltage and current tracings were monitored on a storage oscilloscope (model HM205-3; Hameg Instruments, Anaheim, CA, USA) and were digitized in real time at the sampling frequency of 5–10 kHz with a PCL-818 interface board (Advantech Corp., Taiwan) which was controlled by a 25-MHz Eagletak computer and Snapshot software ver-

sion 3.52 from HEM data Corp (Southfield, Michigan, USA). These records were also simultaneously stored on a digital tape record (model 1202; Biologic Corp., Claix, France), the frequency-response of which is DC-20 kHz with a dynamic of 14 bits. After the experiments, the stored data were processed by a personal computer for analysis, graphing, and archiving. All values are reported as mean ± S.D. Student's paired or unpaired *t*-test was used for statistical analyses. Differences between values were considered significant when $P < 0.05$.

Drug and solutions. All the chemicals were purchased from Sigma Chemicals. The composition of normal Tyrode's solution (300 mOsm/l) was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53. glucose 5.5, Hepes-NaCl buffer 5 (pH 7.4). The composition of hypotonic solution (200 mOsm/l) was as follows (in mM): NaCl 86, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5, Hepes-NaOH buffer 5 (pH 7.4). To measure resting membrane potential, the pipette solution contained (in mM): KCl 130, EGTA 0.1, $MgCl_2$ 2, ATP 3, GTP 0.1 and Hepes-KOH buffer 5 (pH 7.2). In experiments of recording hypotonicity-induced Cl^- current, KCl inside the pipette solution was replaced with equimolar CsCl, and pH was adjusted to 7.2 with CsOH.

3. Results

Tumor histology and the state of HPV DNA in cervical carcinoma. Ten samples of invasive cervical carcinoma were collected in this study. Table 1 listed the clinical, pathological and HPV DNA characteristics of each case. There were 8 cases of squamous cell carcinoma and 2 adenocarcinoma. In addition, 8 were positive for HPV DNA and most of the HPV-positive samples had HPV 16 or 18 DNA.

Characterization of RVD. Fig. 1 illustrates the effect of hypotonicity (i.e., reduction of the bath osmolarity from 300 mOsm/l to 200 mOsm/l) on cell volume. The response can be divided into three phases: (1) an initial and rapid, osmotic swelling reaching a peak 70% above control volume at 300 s; (2) a rapid shrinkage in the following 300 s; (3) a more gradual decrease in cell volume reaching a steady state about 30% above control volume at 900 s. To determine if activation of Cl^- channels was involved in the process of RVD, a Cl^- channel blocker, DIDS (1 mM), was added to the hypotonic bath solution. As shown in Fig. 1, DIDS increased the initial, rapid osmotic swelling, attenuated the shrinkage phase, and inhibited the gradual, slower decrease in cell volume compared with the cells not exposed to DIDS.

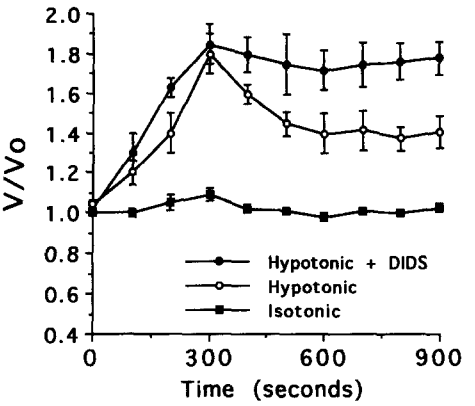

Fig. 1. Time course of cell volume changes following superfusion with isotonic (300 mOsm/l), hypotonic (200 mOsm/l), and hypotonic plus 1 mM DIDS bath solution. The *y*-axis (V/Vo) depicts the ratio of cell volume at time 0 second divided by the cell volume at the indicated times. Each points represents mean ± S.D. ($n = 15$ cells). DIDS: 4,4'-di-iso-thiocyanatostilbene-2,2-disulphonic acid.

Effect of hypotonicity on the membrane currents of primary culture cells of cervical carcinoma. A representative morphology of primary culture cells of cervical carcinoma was shown in Fig. 2. During superfusion with the isotonic Tyrode's solution, primary culture cells had the resting membrane potential of -25 ± 8 mV ($n = 20$ cells for 10 samples) under the current-clamp condition with K^+-containing pipette solution. The cell capacitance and input resistance at the resting potential measured in the voltage-clamp mode were 45 ± 8 pF ($n = 10$) and 1.5 ± 0.7 GΩ ($n = 15$), respectively. After the pipette solution was changed to Cs^+-containing solution and the cells were exposed to the hypotonic solution, the membrane current at the holding potential of +40 mV was obviously shifted in the upward direction by approx. 2.7 nA (Fig. 3, panel A). In addition, when the cell was held at −80 mV, the membrane current was noted to be shifted downward by about −3.8 nA after hypotonic solution was superfused (Fig. 3, panel B). The holding currents returned to control level after hypotonic solution was washed out. Similar results were obtained in repeated experiments.

In order to construct the current-voltage (I-V) relationship, the ramp command pulses were applied at the voltage range between −80 mV and +40 mV with a duration of 100 ms before and after addition of extracellular hypotonic

Table 1
Clinical stage, tumor histology and HPV DNA of ten different samples

Case	Clinical stage	Histopathological type	HPV DNA
A	IIa	Squamous cell carcinoma	16, 18
B	Ib	Squamous cell carcinoma	16
C	IIa	Squamous cell carcinoma	16
D	Ib	Squamous cell carcinoma	Nil
E	IIa	Squamous cell carcinoma	16
F	Ib	Squamous cell carcinoma	18
G	IIa	Squamous cell carcinoma	33
H	Ib	Squamous cell carcinoma	Nil
I	Ib	Adenocarcinoma	18
J	IV	Adenocarcinoma	18

HPV, human papillomavirus.

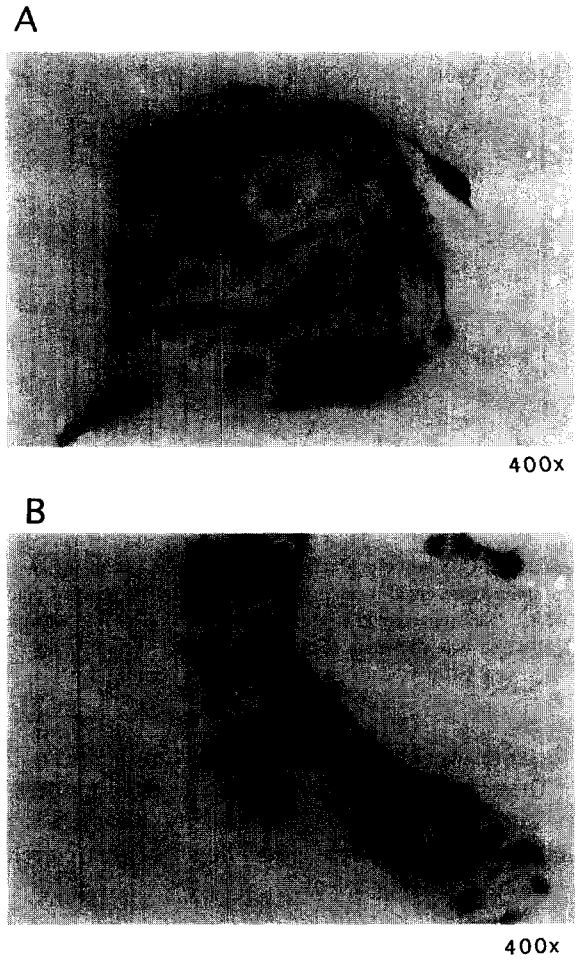

Fig. 2. Primary culture cells from invasive cancer of cervix view in the phase-contrast light microscope (400×).

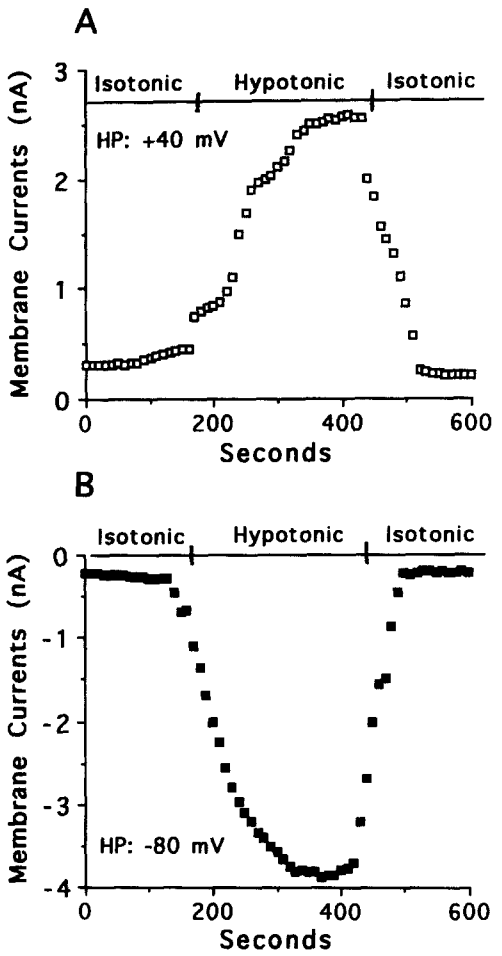

Fig. 3. Effect of hypotonicity (200 mOsm/l) on the holding currents in the primary culture cells of cervical cancer. The cells were held at the level of +40 mV (panel A) and −80 mV (panel B), respectively. The sequences of changes in superfusion solution are illustrated above each panel. HP, holding potential.

solution. In the isotonic bath, these cultured cells have a small background current averaging 0.3 ± 0.2 nA at +40 mV and -0.3 ± 0.1 nA at −80 mV with a slope conductance of 0.32 ± 0.07 nS/pF at +30 mV (Fig. 4, $n = 60$ cells from 10 different samples). The bath medium was then changed into hypotonic solution. Exposure to hypotonicity induced a fast activating and outward rectifying current which was reversed at about 0 mV. Hypotonicity elicited about 11-fold increase in current to 2.5 ± 0.4 nA and -3.6 ± 0.8 nA at +40 and −80 mV, respectively; moreover, the slope conductance at +30 mV was increased by 10-fold to 3.62 ± 0.62 nS/pF. Notable relaxation of the current failed to observe during persisting perfusion with hypotonic solution. In all experiments, V_{rev} for the Cl^- current was relatively constant over time. These results, taken together with the data of tumor histology and HPV positivity, suggest that the activation of volume-sensitive chloride channels in the primary culture cells of cervical cancer is independent of their association with HPV DNA and histopathological type.

Effects of ion replacement and chloride channel blockers on the volume-sensitive Cl^- currents. Increases in

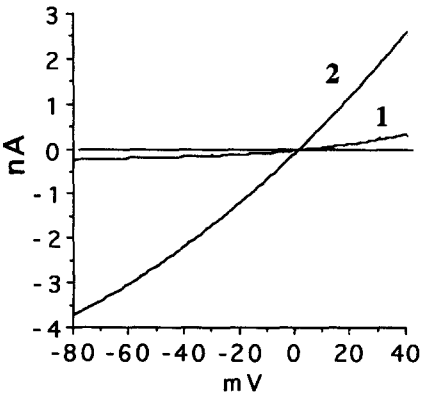

Fig. 4. Volume-sensitive chloride currents in primary culture cells of cervical cancer. The patch pipette contained the 140 mM CsCl-internal solution. The membrane potential was held at −40 mV and the 100-ms ramp pulses from −80 to +40 mV at a rate of 0.2 Hz was applied. 1: membrane current recorded at isotonic bath solution (300 mOsm/l); 2: membrane current recorded after perfusion with hypotonic bath solution (200 mOsm/l).

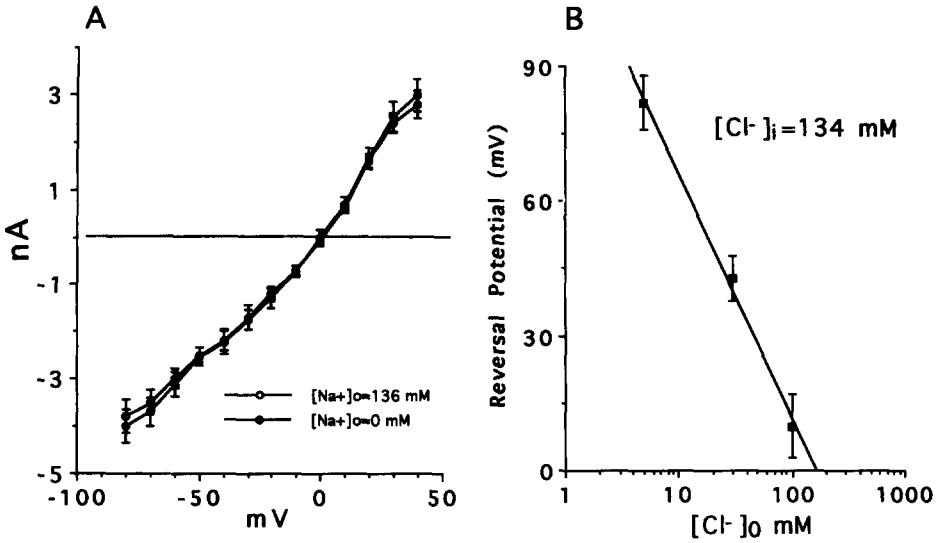

Fig. 5. Na^+-independence and Cl^--dependence of hypotonicity-activated whole-cell current. A: Current-voltage relationship demonstrated the insensitivity of the reversal potential and current amplitude to changes in extracellular sodium. Each point represented mean ± S.D. ($n = 3$). B: The reversal potential for the hypotonicity-activated current at various extracellular Cl^- concentrations ($[Cl^-]o$). Data represented mean ± S.D. ($n = 3$). The line was plotted semilogarithmically and was well fit by the linear regression analysis. Of note, the abscissa was shown in logarithm scale. $[Cl^-]i$: intracellular Cl^- concentration.

inward current at −80 mV and in outward current at +40 mV with no change in current at 0 mV, could also result from activation of a non-selective cation conductance or a leak. To clarify whether this current is carried by chloride ion, ion-replacement and Cl^--blockers studies were done to assess this possibility. As shown in Fig. 5A, replacement of bath Na^+ by choline did not significantly alter the I-V relationship. The external chloride concentration in hypotonic solution was also replaced with equimolar concentration of gluconate. Fig. 5B showed the relationship between reversal potential and extracellular concentration of chloride. When chloride concentration inside the pipette solution was kept at 134 mM and external chloride concentration was reduced by the replacement with gluconate, the V_{rev} for hypotonicity-induced current was noted to be shifted to the more positive level. For instance, when external chloride concentration was reduced to 30 mM and 5 mM, the V_{rev} were significantly changed to 43 ± 5 mV and 82 ± 6 mV, respectively ($n = 3$). This result indicates the strong dependence of changes of ionic currents on extracellular chloride concentration.

Three Cl^- channel blockers were used to test their effects on the volume-sensitive Cl^- currents of primary culture cells: 4,4′-di-iso-thiocyanatostilbene-2,2-disulfonic acid [DIDS], 5-nitro-2-(3-pheny-propylamino)-benzoic acid [NPPB] and 1,9-dideoxyforskolin. As shown in Fig. 6A, 1 mM DIDS produced a remarkable inhibition of volume-sensitive Cl^- currents. This inhibitory effect was rapid onset, dose-dependent and reversible. The EC_{50} required for the inhibitory effect of DIDS was about 150 μM (Fig. 5B). Similarly, NPPB and 1,9-dideoxyforskolin, also reversibly inhibited the volume-sensitive Cl^- currents in a dose-dependent manner. The EC_{50} for NPPB and 1,9-dideoxyforskolin were 120 μM and 50 μM, respectively (Fig. 6B). These results, together with those from ion-replacement studies, suggest that hypotonicity stimulate a Cl^- channel and ruled out the possibility that this current is from a cation flow or a leak.

GTPγS-activated chloride current resembles the volume-sensitive chloride current. To investigate if G-protein mediated the activation of Cl^- channels in cervical carcinoma, the current traces were recorded after 300 μM GTPγS was added in the pipette. Interestingly, the GTPγS-activated Cl^- current was similar to the volume-sensitive Cl^- current induced by hypotonicity (Fig. 7). The current trace for the I-V relationship of GTPγS-activated current also appeared to be outwardly rectifying at the range of −80 mV and +40 mV and reversed at about 0 mV. The slope conductance of GTPγS-activated Cl^- channel at +30 mV was 4.02 ± 0.80 nS/pF ($n = 30$ cells of 6 different samples) which was not significantly different from that of volume-sensitive Cl^- channel (3.86 ± 0.82 nS/pF, $P > 0.05$, t-test).

4. Discussion

This study demonstrates that volume-sensitive chloride currents, leading to RVD, were distinctly activated in primary culture cells of cervical carcinoma. Furthermore, these Cl^- currents whose regulation was linked to G-protein were ubiquitously expressed in samples of various cancer stages and histopathological types, and the presence or absence of HPV DNA. Although activation of Cl^- channels in response to hypotonicity has previously been demonstrated in several epithelial cell lines [3,8,11–14];

however, our present report addresses the possibility of studying volume – sensitive Cl^- channels directly from cervical cancer tissue. Our results show that primary culture cells of cervical cancer develop volume-sensitive chloride currents which were indistinguishable from those in cervical cancer cell lines, in terms of the magnitude of currents, ATP dependency, and reversible inhibition by DIDS, 1,9-dideoxyforskolin, and NPPB. Thus, the preferential activation of volume-sensitive chloride channels in cervical cancer is an inherent characteristic of cervical cancer cells, rather than an acquired phenomenon upon long-term culture.

Cervical cancer is the most frequent cause of disease and death from malignant neoplasms among women in developing countries [15]. It has been known for several years that HPV infection is associated with cervical cancer [16]. However, HPV infection alone is insufficient to initiate malignant transformation of the cervical epithelium [17–19], suggesting that other etiologic factors must be involved in the progression to malignancy.

Our previous observation [4] shows that volume-sensitive chloride currents were induced by hypotonicity in all of the 3 HPV-positive (HeLa, SiHa and CasKi), and 2 HPV-negative (HT-3, and CX) cervical cancer cell lines investigated (4 squamous cell carcinoma, 1 adenocarcinoma), but not in normal cervical cells. The present study further provides six lines of evidence to suggest that

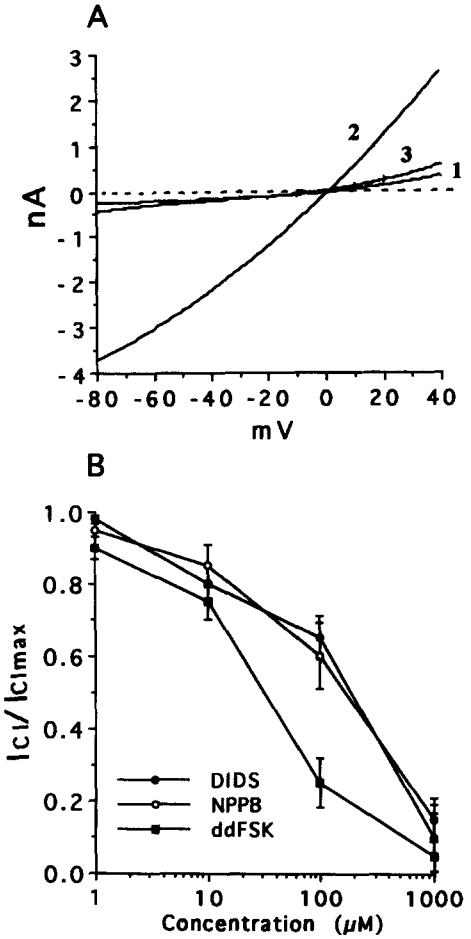

Fig. 6. The volume-sensitive Cl^- current was effectively inhibited by Cl^- channel blockers in primary culture cells of cervical cancer. The patch pipette contained the 140 mM CsCl-internal solution. Panel A shows an example of DIDS inhibition of volume-sensitive Cl^- current. 1: basal membrane current recorded at isotonic medium (300 mOsm/l); 2: current recorded after perfusion with hypotonic medium (200 mOsm/l). 3: current recorded after adding 1 mM DIDS in the hypotonic medium. Panel B illustrates the dose-response relationships of Cl^- channel blockers for the percentage inhibition of hypotonicity-induced current measured at the level of +40 mV. Each point represents mean ± S.D. ($n = 6$). DIDS: 4,4'-di-iso-thiocyanatostilbene-2,2-disulphonic acid; NPPB: 5-nitro-2-(3-pheny-propylamino)-benzoic acid; ddFSK: 1,9-dideoxyforskolin.

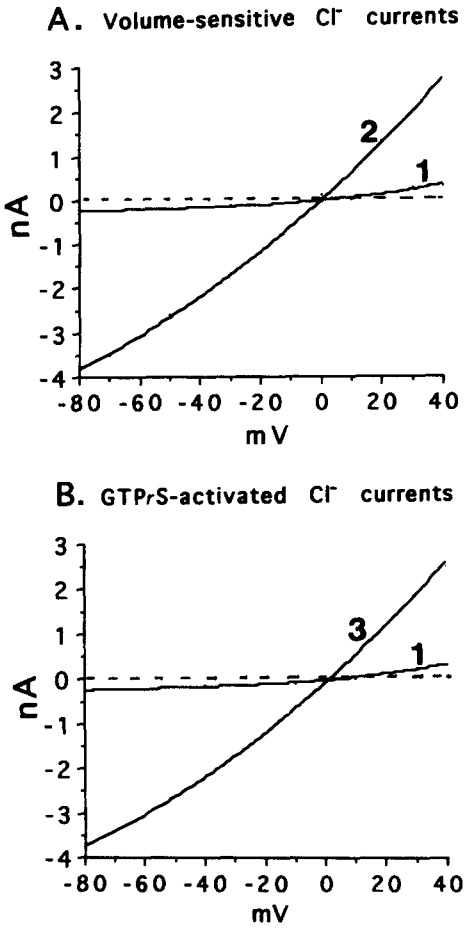

Fig. 7. GTPγS-activated chloride current resembles the volume-sensitive chloride current in the primary culture cells of cervical cancer. The patch pipette contained the 140 mM CsCl-internal solution. The membrane potential was held at −40 mV and the 100-ms ramp pulses from −80 to +40 mV at a rate of 0.2 Hz was applied. 1: membrane current recorded at isotonic medium (300 mOsm/l); 2: membrane current recorded after perfusion with hypotonic medium (200 mOsm/l). 3: membrane current recorded at isotonic medium with 300 μM GTPγS inside the pipette. The current trace for the I-V relationship of GTPγS-activated current also appeared to be outwardly rectifying at the range of −80 mV and +40 mV. The slope conductance of GTPγS-activated Cl^- channel at +30 mV was 4.02 ± 0.80 nS/pF ($n = 30$ cells of 6 different samples) which was not significantly different from that of volume-sensitive Cl^- channel (3.86 ± 0.82 nS/pF, $P > 0.05$, t-test).

volume-sensitive Cl^- channels, leading to RVD, are present in the primary culture cells of cervical cancer. The expression of volume-sensitive Cl^- channels is independent of cancer clinical stage, histopathological type and HPV DNA. Furthermore, G-protein may be linked with the activation of volume-sensitive Cl^- channel in cervical cancer. First, 10 samples of cervical cancer, with different histological type, clinical stage and HPV DNA, were collected in this study. Second, the significant change in ionic current was observed when primary culture cells from 10 different samples exposed to hypotonic solution and this effect was reversed by returning the cells to isotonic medium. Third, the Cl^- permeability through this volume-sensitive ionic conductance was confirmed by the results that the reversal potential derived from current traces of the I-V relationship of hypotonicity-induced current was shifted according to the change in Cl^- equilibrium potential. Fourth, the Cl^- channel blockers (DIDS, NPPB, 1,9-dideoxyforskolin) reversibly inhibited the volume-sensitive Cl^- current in a dose-dependent manner. Fifth, GTPγS-activated chloride current resembles the volume-sensitive chloride current. Sixth, DIDS increased the initial, rapid osmotic swelling, attenuated the shrinkage phase, and inhibited the gradual, slower decrease in cell volume, compared with the cells exposed to hypotonic solution without DIDS. These observations also suggest that elevated expression of volume-sensitive chloride channels might be associated with human cervical carcinogenesis. The precise association of these channels with human cervical carcinogenesis is currently under investigation.

It is noteworthy that G-protein may be linked with the activation of volume-sensitive Cl^- channel in cervical carcinoma. Such a phenomenon has also been observed in the human endothelium [20] but not in *Xenopus* oocytes [21]. Further investigation should be done to elucidate the intracellular signal transduction of activating volume-sensitive chloride channels in cervical cancer cells.

In conclusion, this study presents the finding that the ATP-dependent volume-sensitive Cl^- channel, leading to RVD, can be observed in the primary culture cells of cervical cancer. This type of Cl^- channel may be associated with G-protein and is independent of cancer stage, tumor histology and HPV infection.

Acknowledgements

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